HONTORIA ET AL.--- EFFECT OF KETOCONAZOLE ON *ICHTHYOPHONUS*

**Ketoconazole Inhibits the Growth and Development of *Ichthyophonus* sp.
(Mesomycetozoa) *in Vitro***

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21 ABSTRACT.

22 **Key words.** Teleostei, treatments.

23 The *in vitro* effect of the azol-derivative antifungal Ketoconazole (KZ) on the morphology,
24 growth and development of *Ichthyophonus* sp, was studied. KZ was delivered to culture
25 medium using liposomes (L) or a lipid emulsion (E). Five different KZ doses (5, 50, 100, 200
26 and 400 µg/ml) were assayed with both L and E formulations. Controls consisted of MEM-10
27 alone (C-MEM) or containing the amounts of L or E equivalent to those used in the KZ100
28 and KZ400 treatments (100L, 400L, 100E and 400E, respectively). Clear morphological
29 alterations were observed by light and electron microscopy in the MEM-cultured organisms
30 receiving KZ formulations, specially with KZ400L preparations, both at 7 and 14 days post-
31 inoculation. KZ also inhibited *Ichthyophonus* growth in MEM and its effect was statistically
32 significant with respect to the corresponding controls. KZ also had an inhibitory effect on
33 subsequent *Ichthyophonus* germination in EFSA medium, which was more evident for L
34 formulations when the organism was treated for 7 days and for E formulations in the 14 days
35 treatment. The obtained results endorse the potential use of KZ for the treatment for
36 ichthyophonosis and the interest of confirming it using *in vivo* assays.

Ichthyophonus is an obligate parasite with a wide host spectrum, including some freshwater or anadromous fish and numerous marine fish (McVicar, 1998). The taxonomic position and identity of *Ichthyophonus* have been very controversial since its first description from *Salmo trutta* by Hofer (1893). Firstly considered as a member of Protozoa (Caullery and Mesnil 1905), the organism was ascribed to Fungi by Plehn and Mulsow (1911), but this ascription was further questioned (reviewed in McVicar 1998). Molecular studies have demonstrated that *Ichthyophonus* and other related microbes constitute a phylogenetic group in the boundaries of the animal-fungal divergence, which has been referred to as the DRIP clade (Ragan et al. 1996; Spanggaard et al. 1996). *Ichthyophonus* was later ascribed to the class Mesomycetozoa (Mendoza et al. 2002) and this class was included within Opisthokonta and not in Fungi in the new classification of protists (Adl et al. 2005).

Information on treatment and control methods for ichthyophonosis is very scarce. Some treatments suggested for the first infection steps, such as fenoxetol and paraclorofenoxetol or antibiotics (Van Duijn 1956) proved not to be very effective. Therefore, prophylactic measures such as food pasteurisation (McVicar 1982) or disinfection (Hersheberger, Pacheco and Gregg 2008) were suggested. *In vitro* studies demonstrated the inhibitory activity of the antifungal ketoconazole (KZ) on *Ichthyophonus* growth (Chauvier and Mortier-Gabet 1982). The preliminary *in vitro* and *in vivo* studies of Franco-Sierra (1994) also pointed to a promising effect of KZ, especially when vehiculated in liposomes. KZ is an azol-derivative antifungal agent with a broad spectrum of activity against both superficial and systemic micosis. Its antifungal action is related to the inhibition of cytochrome P-450-dependent demethylation of lanosterol in the biosynthetic pathway of ergosterol in fungi (Vanden Bossche et al. 1988). However, it also affects a number of mammalian cytochromes, and thus can cause liver damage through inhibition of NADH oxidase and thus mitochondrial activity (Rodríguez and Acosta 1996).

Liposomes, as vesicles formed by polar lipids in bilayer arrangements enclosing aqueous compartments, can be manipulated to encapsulate different substances that are isolated from the surrounding medium. Liposomes were first proposed and tested as a drug delivery system more than 30 years ago. Since then, the design of constructs for use in the treatment and prevention of disease has substantially improved (Gregoriadis 1995; Torchilin 2005) and a reduction of toxicity of several drugs when encapsulated in liposomes has been reported (Mehta 1996). Consequently, the use of liposomes as delivering agent for KZ can provide different advantages related to the protection against the drug toxicity, and to the improvement of the treatment efficiency due to the facility of the liposomes to reach the cells and the intracellular pathogens.

We have reported the finding of an *Ichthyophonus* sp. from grey mullets and other marine fish from the Mediterranean area, and provided data on the infection in different mariculture systems (Franco-Sierra, Sitjà-Bobadilla and Alvarez-Pellitero 1997). A detailed morphological study, including ultrastructural data has also been presented (Franco-Sierra and Alvarez-Pellitero 1999). The available data on the KZ properties and the advantages of liposome delivering prompted us to afford in depth studies on the effect of KZ on this Mediterranean ichthyophonosis, using *in vitro* and *in vivo* methods. In the present work, the effect of the drug on the growth of *Ichthyophonus* cultured *in vitro* is evaluated. In addition, the effect of the treatment on the morphology of cultured stages is studied at light and transmission electron microscopes.

MATERIAL AND METHODS

Preparation of liposomes and ketoconazole (KZ)-formulations. Five different KZ doses (5, 50, 100, 200 and 400 µg/ml) were prepared in MEM-10 (see below). Two different

KZ formulations were used to deliver the drug in the culture medium, liposomes (L) or an equivalent lipid emulsion (E). Thus, ten KZ formulations (KZ5L, KZ50L, KZ100L, KZ200L, KZ400L, KZ5E, KZ50E, KZ100E, KZ200E and KZ400 E) were assayed. Controls consisted of MEM-10 alone (C-MEM) or containing the amounts of L or E equivalent to those used in the KZ100 and KZ400 treatments (100L, 400L, 100E and 400E, respectively) (Table 1).

Liposomes were prepared with phosphatidylcholine extracted from egg yolk (EPC) purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama USA). Palmiticic (16:0, 34% of total fatty acids) and oleicic (18:1, 31%) acids are the main fatty acids of EPC. It also contains 18% of linoleic acid (18:2). Cholesterol (CHO), purchased from Sigma-Aldrich Química S.A. (Alcobendas, Madrid, Spain), was included as a membrane stabilizer, and stearylamine (ST), a polar derivative from the stearic acid also from Sigma-Aldrich Química S.A., as charge to prevent the aggregation. In the case of the treatment formulations, 20% w/w of ketoconazole (Acofarma, Barcelona, Spain), was also incorporated to the liposome composition. The liposomes under the form of multilamellar vesicles (MLV) were prepared by the method proposed by Bangham et al. (1965), but using MEM-10 as the aqueous phase. The lipid mixture was dried under nitrogen flux in a thin layer on the bottom of a flask and rehydrated with the aqueous phase during one hour by vortexing frequently until an homogenous suspension was achieved. The final composition of the lipid mixture for the mother preparation used in the liposomes for treatment doses was: EPC: KZ: CHO: ST (56:20:19:5 w/w), and in the control liposomes without KZ: EPC: CHO: ST (76:19:5 w/w). This mixture was then rehydrated with MEM-10 and used in the necessary amounts to reach the experimental doses in each culture well (see below). The lipid emulsions were prepared emulsifying the triacylglyceride triolein (TON) with Tween 80 (polysorbate 80), both acquired from Sigma-Aldrich Química S.A., in MEM-10, using an IKA Ultra-Turrax tissue disruptor (IKA Labortechnik, Staufen, Germany) at high lipidic concentration in order to

improve the stability. The final composition of this mother emulsion was: MEM: TON: Tween80 (89.6:7.1:3.3 v/v). A similar mother emulsion was prepared for the treatment emulsions. In this case, KZ was added to achieve a final concentration of 4 mg/ml. Both emulsions were then diluted with MEM in each culture well to achieve the appropriate experimental doses. The different concentrations of the liposomes or emulsion components in each treatment well are also shown in Table 1.

Culture media. The organism was cultured in Eagle's minimum essential medium (Sigma M5775) (MEM) at pH 7.2 supplemented with HEPES (Gibco) (20 mM), 10 % foetal bovine serum (FBS) (Sigma F4010) (MEM-10) and gentamycin (50 $\mu\text{g}.\text{ml}^{-1}$). Earle's fish saline agar (EFSA), prepared as in Rand and Cone (1990) and supplemented with 10 % FBS, was used to evaluate germination.

***Ichthyophonus* source.** The *Ichthyophonus* used in this study was aseptically taken and isolated from organs of naturally infected mullets suffering an epizootic in August 1990 (Franco-Sierra et al., 1997). Since the original isolation, it has been kept *in vitro* through serial passages in 100 ml bottles containing MEM-10 (see above) and under the conditions selected in a previous study (Franco-Sierra and Alvarez-Pellitero, 1999) (pH 7.2 and 14 °C). *Ichthyophonus* from routine cultures was replicated in the same medium and conditions in T-25 flasks to obtain the amount necessary for the assays.

Evaluation of growth. Tests were performed in six well plates containing 3 ml of MEM-10 per well with the appropriate amounts of the corresponding mother preparations to get the necessary concentrations of each treatment, or MEM-10 alone (C-MEM). The resulting concentrations of each component of E or L in the corresponding control and KZ formulations are detailed in Table 1. Each well was seeded with 100 μl of *Ichthyophonus* culture containing 1.58×10^6 spores/ml. Three replicates per treatment were incubated at 14°C during 7 days and the other three replicates during 14 days. The development of the organism

was followed using an inverted microscope ($\times 100$, Nikon Diaphot-TMD). At the end of each incubation period, the *Ichthyophonus* stages present in each well were collected and the number of viable stages was counted in a Neubauer chamber using the eosin-exclusion method. Part of this material was used to further evaluate germination (see below). Samples were also taken for fresh examination by light microscope (LM) using a Leitz Dialux 22 microscope. For transmission electron microscope (TEM studies), samples from 14 days cultures corresponding to each treatment were pelleted at 700 g for 5 minutes at 4°C. Pellets were fixed in 2.5 % v/v glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2, 4 °C) for 12 h., postfixed in 0.1% (w/v) cacodylic OsO₄ for 2 h, dehydrated through a graded ethanol series and embedded in Spurr' resin (Spurr 1969). Ultrathin sections were double stained with aqueous uranyl acetate and lead citrate (Reynolds 1963). Some grids were stained by the OTO technique for lipids (Seligman et al. 1966)). The sections were studied in a Philips CM 200 or a Hitachi AD 600 transmission electron microscopes, operating between 60 and 75 kV.

Evaluation of further germination. To evaluate the further development of *Ichthyophonus* after 7 or 14 days of treatment, an equal number of *Ichthyophonus* viable stages from each treatment and each incubation period were seeded by triplicate on Petri dishes containing EFSA and incubated at 14 °C. The volume to be seeded in each case was deduced from the counting data indicated above and was calculated to obtain 7.75×10^4 stages per plate. Growth was evaluated as the number of colony forming units (CFU)/ml at 14 days post-seeding. The development of the organism was followed using an inverted microscope as above.

Statistical analysis. The number of viable spores or organism-units per ml grown in MEM (mean of the three replicates) in each treatment after 7 and 14 days was compared using two separate one-way ANOVA tests with the Brown-Forsythe transformation in search of differences among treatments followed by the Games-Howell's test for multiple mean

comparisons when appropriate. Both tests are robust in cases of variance heteroscedasticity, which have been the case in our study due to the high dispersion that these traits show. Differences in the number of CFU/ml observed in EFSA (mean of the three replicates) after incubation periods of 7 and 14 days were also analysed with the same tests.

RESULTS

Morphological studies. The effect of the KZ treatment on *Ichthyophonus* structures was evidenced at both LM and TEM. At LM, spores in the different stages of growth as well as the typical development (division, budding) were seen in C-MEM (Fig. 1). No appreciable changes were induced by the lowest dose (KZ5), but both the spore morphology and the development were altered starting with the KZ50 dose and damage clearly increased with higher doses. Changes consisted mainly of a decrease in the number of dividing and budding spores and an alteration of the spore structure, i.e. retraction and densification of the cytoplasmic material. Such alterations were evident with KZ100 dose and more pronounced with KZ400 dose, being the effect more intense with L (Fig. 2) than with E preparations (Fig. 3). The addition of 100L and 400L alone also induced some alterations in the spores (Fig.4), not or scarcely observed with 100E and 400E (Fig. 5). The noxious effect of KZ was already observed at 7 days post-inoculation (p.i) and was more pronounced at 14 days p.i.

To more accurately evaluate the effect of KZ on *Ichthyophonus* stages, a TEM study was carried out with the material from the different treatments collected at 14 days p.i. In addition, the ability of the organism to germinate was evaluated after seeding in EFSA. In the TEM study, spores cultured in C-MEM had the typical structure (Fig. 6, 7), with abundant nuclei, some endospores, and a well defined wall. The cytoplasm was densely packed with ribosomes and abundant glycogen. Mitochondria, endoplasmic reticulum some vacuoles and

several lipid droplets (as confirmed with OTO staining) were also observed. The addition of KZ produced appreciable effect on spores from the KZ100 dose on. With KZ100E, the number of nuclei was substantially reduced indicating a decrease in cell activity. This effect was more pronounced with KZ400E. In some spores the cytoplasm became amorphous (Fig. 8) and a destruction of the cells contents occurred, though abundant lipidic vesicles were still evidenced by OTO staining (Fig. 9). The addition of KZ100L and KZ400 L induced even more evident alterations of the spores, consisting of condensation of the cytoplasmic contents in some thick-walled spores, or destruction of the cytoplasmic contents and wall (Fig.10). As observed by LM, the addition of 100L and 400L produced also alterations in some spores, mainly condensation of the cytoplasm contents (Fig. 11). Spores maintained in 100E and 400E, did not show such alterations but abundant lipidic droplets (Fig. 12).

Differences in the germination of *Ichthyophonus* in EFSA were observed after 14 days of KZ treatments with respect to controls. *Ichthyophonus* previously cultured in C-MEM showed the typical germination pattern, with formation of long septate hyphae, which, after branching, developed spores in the bulbous tips (Fig. 13). In contrast, many spores previously cultured in KZ100E were not able to germinate (Fig. 14) and no germination was observed in KZ100L (Fig. 15), KZ400E (Fig. 16) and KZ400L (Fig. 17) samples.

Evaluation of growth in MEM-10 and further germination in EFSA. At 7 days p.i., an stimulating effect of lipid addition on *Ichthyophonus* growth was observed in both control L and E treatments with respect to C-MEM, though the difference was statistically significant only for 400L and 400E. Therefore, the effect of KZ treatment must be compared with the corresponding controls with added liposomes or lipids. Thus, the addition of KZ significantly inhibited the growth with respect to L and E controls even at the lowest dose (5KZ) (Fig. 18A). At 14 days of treatment, the effect of the of L or E alone on the growth was slight, and no statistically significant differences were detected with respect to C-MEM. An inhibitory

effect of KZ treatment was also detected, but differences were statistically significant only for the highest doses of E treatments, KZ200E and KZ400E (Fig. 18B). A quite good dose/effect correlation was generally observed.

The further viability of spores was evaluated in EFSA. The germination of *Ichthyophonus* that have been kept for 7 days in KZ100E, KZ200E and KZ400E was significantly lower than in the corresponding E controls and in the C-MEM. At this time, most L treatments had significantly lower values than those of C-MEM (Fig. 19A). The effect of KZE was more evident for *Ichthyophonus* that have been kept for 14 days in the corresponding treatments, as the number of CFU was significantly lower than in E controls for all the doses assayed, and also with respect to C-MEM for the highest doses (Fig. 19B). No significant differences were found at this time between treated and untreated L groups. However, the CFU number was significantly lower for the three highest KZ doses (100L, 200L and 400L) with respect to C-MEM (Fig. 19B).

DISCUSSION

The obtained results demonstrated that KZ inhibited the growth and development of *Ichthyophonus* cultivated *in vitro*. The noxious effect was higher when KZ was delivered in liposomes than when incorporated in the lipid emulsion. Both the growth kinetics and morphological studies evidenced that KZ has a clear effect on this organism and that this effect is influenced by the delivery system. To check the possible advantages of liposomal (L) delivering, KZ had to be incorporated to the culture medium using a lipid emulsion (E) vehicle, as the drug is not hydro soluble. Multilamellar vesicle (MLV) liposomes can encapsulate lipophilic materials as KZ more readily than unilamellar liposomes. The non polar core of the multiple MLV membranes offers more encapsulation space for KZ. The

positive charge afforded by the sterylamine grants a repulsive force between the different membranes that prevents liposome aggregation.

The addition of both L and E alone stimulated the growth and development of *Ichthyophonus* in MEM at 7 days p.i., as the number of viable units was higher, both in L and E controls than in C-MEM. In contrast, this effect was negligible at 14 days p.i. Lipid emulsion addition did not induce important morphological changes, apart from the increasing of lipidic inclusions in the cytoplasm, but the presence of L induced some changes in the organism. It seems that this additional lipid supply favours the growth of *Ichthyophonus*, at least initially. However, such effect was not so clear at longer term, mainly for L preparations, as germination was lower than in C-MEM both after 7 and 14 days in such condition. For E preparations, germination was better for 100E than for 400E. Such results in control L and E formulations seem to indicate that an excess of lipids might be counterproductive and overpass the clear lipasic activity that was demonstrated in previous metabolic studies of *Ichthyophonus* (Franco-Sierra 1994). The effect of L on *Ichthyophonus* growth after 14 days of treatment and in germination at both times might be due to some of the components used in its preparation. The cholesterol concentration in C400L is 0.376 mg/ml, which could be excessively high for a good performance of the organism, as in the case of phosphatidylcholine (1.92 mg/ml). However, stearylamine probably has no effect at the used concentrations, and has been reported as a liposome component improving some effects as the DNA transfection to eukaryotic cells (Wang, Jin and Lin, 1996).

Both KZL and KZE formulations produced a decreasing in the *Ichthyophonus* growth with respect to the corresponding L and E controls, though this effect was statistically significant only for the highest doses. Such effect was confirmed by the results of spore germination, mainly in the case of KZE formulations. The antifungal action of KZ and other imidazol derivatives is related to the inhibition of the biosynthesis of ergosterol, a major sterol

in fungi. As fungal sterols are structurally distinct from their mammalian counterparts, their synthesis has been a major target for antifungal drug development (Oehlschlaeger and Czyżewska, 1992). Like cholesterol in higher organisms, ergosterol is a component of membranes and other cellular organelles in fungi and other microorganisms. However, it has a different biosynthetic pathway due the presence of a Δ -24(25)-sterol methyltransferase enzyme catalysing the incorporation of a methyl group in the side chain of the steroid nucleus, which is absent in the mammalian cells (Barrett-Bee and Ryder, 1992). The proposed mechanism of action is the inhibition of cytochrome P-450-dependent demethylation of lanosterol to ergosterol by the 14- α -demethylase system. Inhibition of ergosterol synthesis may result in direct damage to the fungus. Indirect injury can also be produced by accumulation of 14- α -methyl sterols. Other important enzymes may also be affected (Feldman 1986; Vanden Bossche et al. 1988). Although the mechanisms involved in the observed action of KZ on the *Ichthyophonus* growth and development are unknown, the participation of the inhibition of cytochrome P-450 enzymes as described in fungi cannot be disregarded. As a matter of fact, an effect of imidazoles on cytochrome P450-related biotransformation processes has been described in other systems and even in mammals (Fink-Gremmels 2008), resulting in the inhibition of the mitochondrial functions (Feldman 1986; Rodriguez and Acosta 1996). *Ichthyophonus* was initially classified within Fungi, but currently is considered to belong to Mesomycetozoa. Scarce data are available on the metabolic pathways of *Ichthyophonus*. Franco-Sierra (1994) found slight phosphatase and clear lipase activities in this organism, but the lipidic composition of the membranes, and thus the presence of ergosterol is unknown.

The morphologic alterations observed in *Ichthyophonus* treated in KZ-containing MEM-formulations consisted mainly of the loss of refringent bodies, darkening and condensation of the cytoplasm and alterations of the membrane, and were related with the

impairment of cellular activity. The effect on further germination in EFSA plates was dramatic for KZ100L, KZ400L and KZ400E formulations, though some typical germination was still observed with KZ100E treatment. The ultrastructural study, corresponding to the material treated in KZ-containing MEM-formulations during 14 days, demonstrated the loss of cytoplasmic integrity, which appeared completely withered in some spores, in contrast with the dense cytoplasmic contents observed in control media. Glycogen granules, very abundant as reserve in control organisms, were very scarce or absent in treated spores, in which, furthermore, very few nuclei, organelles and ribosomes were observed. In addition, different degrees of disorganization were detected in the spore wall. Ultrastructural changes induced by KZ have been reported in other *in vitro* cultured parasites, such as *Leishmania*, consisting of the appearance of large multivesicular bodies, increased amount of lipid inclusions, and alterations in the distribution and appearance of mitochondrial cristae, accompanied by strong phosphatase acid activity (Vannier-Santos et al. 1995). Another azole derivate, itraconazole, provokes pellicle disruption in *Toxoplasma gondi* and affects the parasite division by endodyogeny (Martins-Duarte, de Souza and Vommario 2008). Different alterations have been described in *Leishmania amazonensis* treated with sterol methenyl transferase inhibitors, mainly associated with mitochondria (Rodrigues et al., 2007). In addition, alterations in organelles such as nucleus, endoplasmic reticulum and Golgi complex have also been described in other parasites with different drugs related or not with ergosterol byosynthesis (de Carvalho et al. 2005; Martins-Duarte et al. 2006; Granthom et al. 2007). The scarcity of organelles in KZ treated *Ichthyophonus* difficults the evaluation of damage at that level.

In conclusion, KZ proved to be effective in inhibiting the growth and development of *Ichthyophonus* sp. *in vitro*. Both delivering systems, liposomes and lipid emulsion can be successfully used to vehiculate the drug. Under the light of the obtained results, further *in vivo* studies are needed to ascertain the potential of KZ as treatment for ichthyophonosis .

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Figure legends

Fig. 1-5. Effect of KZ on the *Ichthyophonus* growth in MEM-10. **1.** Normal growth with division and budding in control (C-MEM) cultures. **2, 3.** The effect of KZ is evident in KZ400L (**2**) and KZ400E (**3**) treatments. **4.** Spore morphology is somewhat altered in 400L. **5.** In 400E, spores have a normal appearance and can experience budding. Scale bars = 50 μm .

Fig. 6-12. TEM images of *Ichthyophonus* kept for 14 days in C-MEM or in media with E and L or KZ formulations. **6, 7.** Morphology of spores in C-MEM showing abundant ribosomes, glycogen granules and several nuclei (arrowheads). The presence of lipid inclusions is demonstrated by OTO staining (**7**). **8, 9.** The effect of KZ is observed KZ400 E treatment. The wall structure is altered and the cytoplasm losses its integrity. Lipid inclusions can be abundant, as demonstrated with OTO staining (**9**). Arrowhead points to a nuclei. **10.** The effect of KZ400L is dramatic. The wall is altered and the cytoplasm appears disintegrated, with visible organelles and very scarce nuclei (arrowhead). **11.** Spores in 400L show altered morphology, with disorganization of the wall and cytoplasm. Arrowheads point to nuclei. **12.** Spores in 400E show a normal morphology, though lipidic inclusions are very abundant. Arrowheads point to nuclei. Scale bars: Figs. 6-9, 11-12 = 1 μm ; Fig. 10 = 4 μm .

Fig. 13-17. Germination of *Ichthyophonus* in EFSA medium after maintenance in C-MEM or in media with KZ formulations. **13.** The organism from C-MEM shows the typical germination with formation of dichotomic hyphae. **14-17.** Germination decreases in the organisms previously kept in KZ formulations. Germination is still observed for KZ100E (**14**), but is almost absent for KZ100L (**15**) and specially for KZ400E (**16**) and KZ400L (**17**). Scale bars = 100 μm .

Fig. 18. Number of *Ichthyophonus* viable units (mean $\pm$ SD) present in C-MEM cultures after 7 (**A**) and 14 (**B**) days of treatment in KZ or E and L formulations. Different letters indicate

437 statistically significant differences ($P \leq 0.05$) after the one-way ANOVA tests and the multiple
438 comparisons of means. Solid lines indicate the control (C-MEM) mean values of the
439 triplicates and dotted lines their associated standard deviations.

440 **Fig. 19.** Number of *Ichthyophonus* colony forming units (CFU) (mean $\pm$ SD) germinated in
441 EFSA after previous treatment with the KZ or E and L formulations during 7 (**A**) or 14 (**B**)
442 days. Different letters indicate statistically significant differences ($P \leq 0.05$) after the one-way
443 ANOVA tests and the multiple comparisons of means. Solid lines indicate the control (C-
444 MEM) mean values of the triplicates and dotted lines their associated standard deviations.

Fig. 1-5. Effect of KZ on the Ichthyophonus growth in MEM-10. 1. Normal growth with division and budding in control (C-MEM) cultures. 2, 3. The effect of KZ is evident in KZ400L (2) and KZ400E (3) treatments. 4. Spore morphology is somewhat altered in 400L. 5. In 400E, spores have a normal appearance and can experience budding. Scale bars = 50 µm.
88x172mm (300 x 300 DPI)

Fig. 6-12. TEM images of *Ichthyophonus* kept for 14 days in C-MEM or in media with E and L or KZ formulations. 6, 7. Morphology of spores in C-MEM showing abundant ribosomes, glycogen granules and several nuclei (arrowheads). The presence of lipid inclusions is demonstrated by OTO staining (7). 8, 9. The effect of KZ is observed KZ400 E treatment. The wall structure is altered and the cytoplasm loses its integrity. Lipid inclusions can be abundant, as demonstrated with OTO staining (9). Arrowhead points to a nuclei. 10. The effect of KZ400L is dramatic. The wall is altered and the cytoplasm appears disintegrated, with visible organelles and very scarce nuclei (arrowhead). 11. Spores in 400L show altered morphology, with disorganization of the wall and cytoplasm. Arrowheads point to nuclei. 12. Spores in 400E show a normal morphology, though lipidic inclusions are very abundant. Arrowheads point to nuclei. Scale bars: Figs. 6-9, 11-12 = 1 μ m; Fig. 10 = 4 μ m.

170x252mm (300 x 300 DPI)

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Figs. 13-17. Germination of *Ichthyophonus* in EFSA medium after maintenance in C-MEM or in media with KZ formulations. 13. The organism from C-MEM shows the typical germination with formation of dichotomic hyphae. 14-17. Germination decreases in the organisms previously kept in KZ formulations. Germination is still observed for KZ100E (14), but is almost absent for KZ100L (15) and specially for KZ400E (16) and KZ400L (17). Scale bars = 100 μ m. 142x219mm (300 x 300 DPI)

Fig. 18. Number of *Ichthyophonus* viable units (mean $\hat{A} \pm$ SD) present in C-MEM cultures after 7 (A) and 14 (B) days of treatment in KZ or E and L formulations. Different letters indicate statistically significant differences ($P \leq 0.05$) after the one-way ANOVA tests and the multiple comparisons of means. Solid lines indicate the control (C-MEM) mean values of the triplicates and dotted lines their associated standard deviations.

209x296mm (144 x 144 DPI)

Fig. 19. Number of *Ichthyophonus* colony forming units (CFU) (mean $\pm$ SD) germinated in EFSA after previous treatment with the KZ or E and L formulations during 7 (A) or 14 (B) days. Different letters indicate statistically significant differences ($P \leq 0.05$) after the one-way ANOVA tests and the multiple comparisons of means. Solid lines indicate the control (C-MEM) mean values of the triplicates and dotted lines their associated standard deviations.

209x296mm (144 x 144 DPI)

Table 1. Concentrations of KZ and the other components in each dose of liposome and lipid emulsion formulations used to evaluate *Ichthyophonus* growth in MEM-10 in the microplate wells. KZ: ketoconazole. EPC: egg yolk phosphatidylcholine; CHO: cholesterol; TON: triacylglyceride triolein.

KZ µg/ml	In liposomes				In lipid emulsions		
	Formulations	EPC	CHO	Stearylamine	Formulations	TON	Tween 80
		mg/ml	mg/ml	mg/ml		mg/ml	mg/ml
5	KZ5L	0.014	0.005	0.001	KZ5E	0.076	0.045
50	KZ50L	0.140	0.048	0.012	KZ50E	0.757	0.447
100	KZ100L	0.280	0.095	0.024	KZ100E	1.516	0.894
200	KZ200L	0.560	0.188	0.050	KZ200E	3.040	1.766
400	KZ400L	1.120	0.376	0.100	KZ400E	6.081	3.543
0	C100L	0.380	0.095	0.024	C100E	1.607	0.894
0	C400L	1.520	0.376	0.100	C400E	6.446	3.543